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(54) Title: USE OF MONOCARBOXYLATE TRANSPORTER PROTEIN FOR THYROID HORMONE TRANSPORT

(57) Abstract: The invention provides a use of a monocarboxylate transporter protein or a functional part, derivative and/or analogue thereof for altering transport of a thyroid hormone or a functional part, derivative and/or analogue thereof across a membrane. Said monocarboxylate transporter protein preferably comprises MCT-8. An isolated molecule capable of specifically binding at least part of an MCT protein, at least part of a ligand of an MCT protein, or a nucleic acid encoding said MCT and/or ligand, is also herewith provided. Regulation of the bioavailability of thyroid hormone in a tissue enables interfering with (metabolic) diseases. Hence, the invention also provides pharmaceutical compositions comprising a compound capable of binding said MCT protein, or capable of influencing the binding or transporting of a ligand of said MCT protein. Methods for diagnosis and/or treatment of a disease such as a disorder of thyroid metabolism, non-thyroidal illness, obesity or cardiovascular illness are also provided, as well as bioassays for identifying or detecting a candidate drug capable of binding to or influencing at least part of said MCT protein.

Title: Use of monocarboxylate transporter protein for thyroid hormone transport

The invention relates to the field of endocrinology, more specifically to the field of regulating thyroid hormone bioavailability and activity.

Thyroid hormone is essential for the development and homeostasis of different organs, particularly for the regulation of energy metabolism. The follicular cells of the thyroid gland produce predominantly the prohormone thyroxine (3,3',5,5'-tetraiodothyronine, T4) which has little or no biological activity. T4 is activated by enzymatic outer ring deiodination to 3,3',5-triiodothyronine (T3), which is the most if not only bioactive form of thyroid hormone (1-4). Both T4 and T3 are inactivated by enzymatic inner ring deiodination to the metabolites 3,3',5'-triiodothyronine (reverse T3, rT3) and 3,3'-diiodothyronine (3,3'-T2), respectively (1-4).

The three deiodinases involved in these processes show different catalytic profiles, tissue distributions and regulatory functions. They have recently been identified as homologous transmembrane selenoproteins with their active site exposed to the cytoplasm (1-4).

Additional pathways of thyroid hormone metabolism include sulfation and glucuronidation of the phenolic hydroxyl group by transferases located in the cytoplasm and endoplasmic reticulum of different tissues (2,4).

Thyroid hormone is essential for the development of different tissues, for example the brain, and for the regulation of energy metabolism of a wide range if not all tissues throughout life (5) and regulating its bioavailability or activity in various tissues would enable regulating, influencing or interfering with (metabolic) disease in those tissues.

Considering the central role that is played by thyroid hormone in the basal metabolism of a wide variety of cells in a wide range of tissues, use of

drugs comprising thyroid hormone agonists and/or antagonists will have beneficial effects in many diseases.

Access of plasma thyroid hormone to intracellular receptors and enzymes requires transport across the cell membrane. On the basis of the lipophilic nature of iodothyronines, it was assumed for a long time that they cross the cell membrane by simple diffusion. However, this ignored the highly polar nature of the alanine side chain which is a formidable obstacle for membrane passage of iodothyronines. During the last two decades, although none were found, overwhelming evidence has accumulated indicating the involvement of plasma membrane transporters in tissue uptake of thyroid hormone (4,6).

In EP 0982399 we reported that such plasma membrane thyroid transporters indeed exist. In that patent application plasma membrane thyroid transporters are provided, such as polypeptides related to an organic anion transporting peptide. These peptides comprise sodium-dependent taurocholate co-transporting polypeptide or sodium-independent organic anion-transporting peptide. An example of such a polypeptide is rat Ntcp, which is a 362-aminoacid protein containing 7 putative transmembrane domains and 2 glycosylation sites with an apparent molecular mass of 51 kDa (7,9,10). It and its orthologues are only expressed in differentiated mammalian hepatocytes, where it is localized selectively to the basolateral cell membrane (9, 10). It is the major transporter of conjugated bile acids in liver but it also mediates uptake of unconjugated bile acids (9, 10 11). The reported Km values of taurocholate for rat Ntcp vary between 15 and 51 μM (10). Ntcp may also mediate transport of a number of non-bile acid amphipathic compounds, including estrogen conjugates such as estrone 3-sulfate (11).

Another example is rat oatp1 which is a 670-aminoacid protein with 12 transmembrane domains and 2 glycosylation sites with an apparent molecular mass of 80 kDa (8-10). Oatp1 is not only expressed in liver but also in kidney

and brain. Like Ntcp, oatp1 is localized to the basolateral liver cell membrane. Oatp1 is a multispecific transporter mediating the uptake of a wide variety of amphipathic ligands (9, 10, 12-15), including conjugated and unconjugated bile acids, conjugated steroids (*e.g.* estrone sulfate, estradiol 17ß-glucuronide and DHEA sulfate) and other organic anions (*e.g.* the prototypic bromosulfophthalein), but also neutral steroids (*e.g.* aldosterone and cortisol), cardiac glycosides (*e.g.* ouabain) and even organic cations (*e.g.* ajmalinium). Apparent Km values of taurocholate and bromosulfophthalein for rat oatp1 amount to 50 and 1.5 μM, respectively (10). In contrast to Ntcp, transport through oatp1 is not coupled to Na^+. Various members of the oatp transporter family in humans and rats have now been shown to be capable of transporting thyroid hormone (16)

It is an object of the present invention to provide alternative plasma membrane thyroid transporters.

Surprisingly it has been found by the present inventors that a monocarboxylate transporter protein (MCT) is capable of transporting a thyroid hormone across a plasma membrane. This class of proteins is reported to transport monocarboxylates such as lactate and pyruvate (Reviewed in (17)). Until now, no correlation with thyroid hormone transport was known.

The invention provides a use of a monocarboxylate transporter (MCT) protein or a functional part, derivative and/or analogue thereof for altering transport of a thyroid hormone or a functional part, derivative and/or analogue thereof across a membrane. Preferably, said transport is enhanced. When the term "MCT protein" is mentioned in the description, it can also refer to a functional part, derivative and/or analogue of an MCT protein. Likewise, the term "thyroid hormone" as used herein is also meant to comprise a functional part, derivative and/or analogue of said thyroid hormone.

Transport of a thyroid hormone across a membrane, such as for instance a plasma membrane, can for instance be enhanced by providing MCT protein to said membrane. Uptake of said MCT protein in said membrane can increase the concentration of said MCT protein. A higher concentration of MCT can result in increased transport of thyroid hormone across said membrane. An MCT protein can be directly provided to a membrane (preferably a plasma membrane) by administration of said protein to said membrane and/or to a cell comprising said membrane. In one embodiment, essentially pure protein is administered. In another embodiment, said protein is part of a (pharmaceutical) composition. Said composition preferably comprises a suitable carrier and/or adjuvant.

An MCT protein can as well be indirectly provided to a (preferably plasma) membrane, for instance by providing a cell comprising said membrane with a nucleic acid encoding said protein. This can for instance be performed by a gene delivery vehicle. Upon expression of said MCT protein by said cell, the amount of MCT protein in said membrane is increased. Said increased amount of MCT results in enhanced thyroid hormone transport across said membrane.

Transport of a thyroid hormone can also be altered by a molecule capable of specifically binding a monocarboxylate transporter protein or a functional part, derivative and/or analogue thereof, or a ligand of said monocarboxylate transporter protein. Such isolated molecule is therefore also herewith provided. In one embodiment, said molecule comprises a proteinaceous molecule such as a (poly)peptide. A molecule with specific binding properties can be generated and/or identified by methods known in the art. For instance, one can use Pepscan techniques and/or replacement mapping techniques as well as for instance phage-display techniques and/or screening of combinatorial libraries, allowing identification of active sites in a polypeptide sequence.

A molecule capable of specifically binding at least part of an MCT protein, or capable of specifically binding at least part of a ligand of an MCT protein, can for instance act as an antagonist, decreasing transport of thyroid hormone across a membrane by said MCT protein. Alternatively, said molecule can act as an agonist, enhancing thyroid hormone transport. The invention thus provides a use of a molecule of the invention for altering transport of a thyroid hormone or a functional part, derivative and/or analogue thereof across a membrane. Preferably a use of the invention is provided wherein said membrane comprises a plasma membrane.

In one embodiment of the invention transport of a thyroid hormone is altered by a molecule capable of specifically binding a nucleic acid sequence encoding a monocarboxylate transporter protein or a functional part, derivative and/or analogue thereof, and/or encoding a ligand of said monocarboxylate transporter protein or functional part, derivative and/or analogue. Said molecule is therefore also herewith provided. Said molecule may be administered to a cell comprising said nucleic acid sequence encoding MCT and/or a ligand thereof. Said molecule may be administered either directly or indirectly, for instance by administration of a nucleic acid sequence encoding said molecule. Upon administration, said molecule will specifically bind said nucleic acid sequence and, hence, will influence the expression of said MCT and/or said ligand. This results in an altered MCT and/or ligand content of said cell and, as a result, in an altered transport of a thyroid hormone or functional part, derivative and/or analogue thereof. Said altered expression of said MCT and/or said ligand preferably results in an enhanced transport of said thyroid hormone or functional part, derivative and/or analogue.

By a molecule capable of specifically binding a nucleic acid sequence is meant herein a molecule that is capable of distinguishing between related

nucleic acid sequences under stringent conditions. A molecule capable of specifically binding a proteinaceous molecule is defined as a molecule that is capable of distinguishing between different proteinaceous molecules because it has a higher affinity for a specific amino acid sequence of said proteinaceous molecule, a specific conformation of said proteinaceous molecule, etc. Non-specific "sticking" of a molecule is not considered "specific binding".

Once a molecule of the invention, capable of specifically binding at least part of an MCT protein, or a functional part, derivative, analogue and/or ligand thereof, or a nucleic acid encoding therefore, has been generated and/or isolated, a desired property, such as its binding capacity, can subsequently be improved. In case of a proteinaceous molecule of the invention this can for instance be done by an Ala-scan and/or replacement net mapping method. With these methods, many different proteinaceous molecules are generated, based on an original amino acid sequence but each molecule containing a substitution of at least one amino acid residue. Said amino acid residue may either be replaced by alanine (Ala-scan) or by any other amino acid residue (replacement net mapping). Each variant is subsequently screened for said desired property. Generated data are used to design an improved proteinaceous molecule.

In a preferred embodiment said monocarboxylate transporter protein comprises monocarboxylate transporter protein-8 (MCT8). MCT8 was cloned in 1994 by Lafreniere *et al* but its role had never been elucidated. The gene consists of 6 exons coding for a protein with 12 putative transmembrane domains. MCT8 is highly expressed in liver, but also in heart, brain, placenta, lung and kidney. As is illustrated by the examples, MCT-8 shows very good thyroid hormone transporter activity. Transport of thyroid hormone across a plasma membrane appears even to be carried out more efficiently by MCT-8 as compared to currently known Ntcp and oatp transporters. However, other

members of the MCT family such as MCT-1, MCT-2, MCT-3, MCT-4, MCT-5, MCT-6, MCT-7 and MCT-9 are also within the scope of the present invention.

A functional part of a protein is defined as a part which has the same kind of properties in kind, not necessarily in amount. A functional derivative of a protein is defined as a protein which has been altered such that the properties of said molecule are essentially the same in kind, not necessarily in amount. A derivative can be provided in many ways, for instance through conservative amino acid substitution.

A person skilled in the art is well able to generate analogous compounds of a protein. This can for instance be done through screening of a peptide library. Such an analogue has essentially the same properties of said protein in kind, not necessarily in amount.

A functional part of a thyroid hormone is defined as a part which has the same kind of properties of influencing development of a tissue and/or regulating energy metabolism of a tissue in kind, not necessarily in amount. A functional derivative of a thyroid hormone is defined as a proteinaceous molecule which has been altered such that the properties of said molecule are essentially the same in kind, not necessarily in amount. A derivative can be provided in many ways, for instance through conservative amino acid substitution. An analogue of a thyroid hormone has essentially the same properties of a thyroid hormone in kind, not necessarily in amount. Said analogue preferably comprises a prohormone thyroxine 3,3',5,5'-tetraiodothyronine (T4) or a molecule that is derived from said prohormone. Preferably, said molecule is derived from said prohormone by outer and/or inner ring deiodination. In one embodiment said analogue comprises 3,3',5'-triiodothyronine (reverse T3) and/or 3,3'-diiodothyronine (3,3'-T2). As used herein, 3,3'-T2 is also referred to as T2.

In one aspect the invention provides a compound capable of influencing the binding or transporting of a ligand of, or capable of binding to, a plasma membrane polypeptide capable of transporting a thyroid hormone, wherein said polypeptide comprises a monocarboxylate transporter protein or a functional part, derivative and/or analogue thereof, for use as a medicament. In another aspect the invention provides a compound capable of specifically binding a nucleic acid sequence encoding a monocarboxylate transporter protein or a functional part, derivative and/or analogue thereof, or encoding a ligand of said monocarboxylate transporter protein, for use as a medicament. Preferably, said polypeptide is capable of transporting 3,3',5,5'-tetraiodothyronine, 3,3',5-triiodothyronine, 3,3',5'-triiodothyronine and/or 3,3'-diiodothyronine. More preferably, said polypeptide is capable of transporting 3,3',5-triiodothyronine. In another preferred embodiment said polypeptide is capable of transporting 3,3',5,5'-tetraiodothyronine. In a most preferred embodiment, said monocarboxylate transporter protein comprises monocarboxylate transporter protein-8.

A compound as provided by the invention is suitable for use in methods for treating a wide range of disorders, such as disorders of thyroid hormone metabolism, for example related to brain disorders seen with psychiatric disease, to restore or help develop tissue metabolism or development in premature children, to help restore thyroid hormone function in patients wherein the thyroid has been removed or is dysfunctioning, for example due to a malignancy, to help alleviate non-thyroidal illness, to treat obesity or cardiovascular illness, to name a few. In fact, all disorders or diseases wherein the (basal) metabolism of a cell or tissue is affected can be treated with a compound of the invention, which can act as an agonist or as an antagonist. An agonist mainly acts in up-regulating metabolism and an antagonist mainly acts in down-regulating metabolism. In a preferred embodiment, such a compound of the invention comprises a peptide, preferably a synthetic peptide,

or an antibody or other binding molecule or thyroid hormone analogue capable of binding to or influencing or interfering with the binding or transporting of a ligand (for example T3) of MCT. A compound of the invention is preferably capable of binding at least part of MCT, and/or at least part of a ligand of MCT. Optionally a compound as provided by the invention is provided with a carrier known in the art for production of a medicament. Said carrier may be a diluent.

Compounds comprising tissue specific thyroid hormone agonists and/or antagonists as provided by the invention can for example be used in treating obesity, heart failure or (tissue specific) hypo- or hyper-thyroidism. Another example is when tissue specific malignancies such as tumours require up- or down-regulation. Such malignancies can be treated by determining which thyroid hormone transporter is used by the cells in the tissue or malignancy, (for example by biopsy and histochemistry using immunological detection or detection of mRNA expression) and then treating the patient with an agonist/antagonist as provided by the invention which specifically acts through the then determined transporter. Furthermore, compounds comprising (tissue specific) thyroid hormone agonists/antagonists as provided by the invention can be used for the production of a medicament for treating a disorder of thyroid metabolism, non-thyroidal illness, obesity or cardiovascular illness.

Thus, the invention provides a use of a compound of the invention for the production of a medicament for the treatment of a thyroid hormone related disorder, non-thyroidal illness, obesity or cardiovascular illness.

A pharmaceutical composition comprising a compound of the invention and a suitable carrier is also herewith provided. Said carrier preferably comprises a suitable adjuvant such as for instance Specol or a double oil emulsion. Moreover, said compound may be coupled to a solid carrier, such as keyhole limpet hemocyanin (KLH) or an immunogenic conjugate of a protein such as ovalbumin. Such pharmaceutical compound is particularly suitable for treating a disorder such as a disorder of thyroid metabolism, non-thyroidal

illness, obesity or cardiovascular illness. In one embodiment the invention therefore provides a method for treating a disorder such as a disorder of thyroid metabolism, non-thyroidal illness, obesity or cardiovascular illness, comprising administering a compound or a pharmaceutical composition of the invention to an individual suffering from said disorder. Said compound or pharmaceutical composition can for instance be administered to an individual orally, by aerosol or as a suppository. Alternatively, said compound can be administered with aid of gene therapy, involving administration of a nucleic acid encoding at least part of said compound, and expression and translation of said nucleic acid, preferably in a host cell. Said nucleic acid for instance comprises DNA or RNA. Methods for the preparation and administration of a compound or pharmaceutical composition are known in the art, as well as suitable carriers for pharmaceutical compositions. Likewise, methods for gene therapy are known by the person skilled in the art. In the art, many vectors and protocols are provided allowing the person skilled in the art to perform an optimal therapy for each application.

A use of at least a functional part of a monocarboxylate transporter protein, or of a nucleic acid encoding at least a functional part of a monocarboxylate transporter protein, in a method for treating a disorder such as a thyroid hormone related disorder such as a disorder of thyroid hormone metabolism, non-thyroidal illness, obesity or cardiovascular illness is also herewith provided. In one embodiment, said use comprises gene therapy.

In yet another aspect the invention provides a method for altering transport of a thyroid hormone or a functional part, derivative and/or analogue thereof across a membrane comprising providing said membrane with a monocarboxylate transporter protein or a functional part, derivative and/or analogue thereof, and/or with a molecule capable of specifically binding a monocarboxylate transporter protein or a functional part, derivative and/or analogue thereof, or a ligand of said monocarboxylate transporter protein,

and/or with a molecule capable of specifically binding a nucleic acid sequence encoding a monocarboxylate transporter protein or a functional part, derivative and/or analogue thereof, or encoding a ligand of said monocarboxylate transporter protein or functional part, derivative and/or analogue. As has been outlined before, an MCT protein and a molecule capable of specifically binding at least part of an MCT protein or at least part of a ligand of an MCT protein, or a nucleic acid encoding said MCT and/or ligand, are particularly suitable for altering thyroid hormone transport across a membrane, preferably across a plasma membrane. In a preferred embodiment said MCT protein comprises MCT-8.

An MCT protein or a nucleic acid encoding at least a functional part of an MCT protein, preferably an MCT-8 protein, can be used in a method for detecting a pharmaceutical compound. The identification of MCT protein involved in the uptake of thyroid hormone is not only important in the study of the regulation of these processes in health and disease, but it also provides for the detection and/or development of thyroid hormone agonists and antagonists which are beneficial in the treatment of conditions such as obesity and cardiovascular diseases. Ligands or blockers of MCT proteins are important candidate drugs for the development of pharmaceutical compositions acting as a (tissue specific) agonist or antagonist of thyroid hormone activity, which are herewith provided. Methods to identify and/or generate a ligand or blocker of a protein, such as an MCT protein, are known in the art. One can for instance use an assay allowing thyroid hormone and a candidate drug compound to compete for transport across a membrane by an MCT protein. One can also provide a host cell with a nucleic acid sequence encoding at least part of said protein and allow for expression of said nucleic acid sequence. Transport of thyroid hormone in the presence of a candidate drug compound across the membrane of said host cell can subsequently be determined. The invention therefore provides a use of at least a functional part of a monocarboxylate

transporter protein, or a nucleic acid encoding at least a functional part of a monocarboxylate transporter protein, in a method for detecting and/or generating a pharmaceutical compound. A preferred embodiment provides a use of the invention wherein said monocarboxylate transporter protein comprises monocarboxylate transporter protein-8.

In one embodiment, such ligands or blockers comprise a peptide derived from a nucleic acid or fragment thereof encoding an MCT protein. To derive peptides which act as ligand (agonist) or blocker (antagonist) is a skill known in the art, for example, one can use Pepscan techniques or replacement mapping techniques as well as for example phage-display techniques and screening of combinatorial libraries, allowing identification of active sites in a polypeptide sequence. In particular, the invention provides a peptide at least comprising an active site capable of binding to or influencing or interfering with the binding and/or transporting of a ligand (preferably of a thyroid hormone nature or functionally equivalent thereto) of a thyroid hormone binding site or part thereof, of an MCT protein, preferably MCT-8.

Furthermore, the invention provides a (synthetic) antibody or other binding molecule specifically directed against an MCT protein, preferably MCT-8, or at least binding to or interfering with the binding of a ligand of a thyroid hormone binding site or parts thereof, of said MCT protein. Generating antibodies or other binding molecules is a skill available in the art, and can be done with classical immunological techniques as well as for example with phage-display techniques.

The invention also provides a bioassay or method to identify said candidate drug agonists or antagonists, for example for use in a pharmaceutical composition for treating obesity, heart failure or (tissue specific) hypo- or hyper-thyroidism. Candidate drugs, often first selected or generated via combinatorial chemistry or comprising a peptide as provided by

the invention, can now be tested and identified using a method provided by the invention. Such a candidate drug or compound can for example be tested on and selected for its effect on T3 uptake by an MCT protein. For use in brain cells, where T3 is autonomously produced, a candidate drug or compound can for example be tested on and selected for its effect on T4 uptake as a precursor for T3. As for example can be seen in several of the figures in the description, the invention provides methods and means to measure thyroid hormone cellular uptake by an MCT protein, and regulation thereof.

The invention thus provides in one aspect a bioassay to identify or detect a candidate drug capable of binding to or influencing a plasma membrane polypeptide capable of transporting a thyroid hormone, wherein said polypeptide comprises a monocarboxylate transporter protein or a functional part, derivative and/or analogue thereof.

In a preferred embodiment, the invention provides a bioassay of the invention using at least a functional part of a monocarboxylate transporter protein, a nucleic acid encoding at least a functional part of a monocarboxylate transporter protein, a molecule capable of specifically binding at least part of said monocarboxylate transporter protein or a ligand thereof, a cell comprising said nucleic acid and/or a cell comprising at least part of said monocarboxylate transporter protein.

In a most preferred embodiment a bioassay of the invention is provided wherein said monocarboxylate transporter protein comprises monocarboxylate transporter protein-8.

The invention also provides an isolated or recombinant monocarboxylate transporter-8 protein or a functional part, derivative and/or analogue thereof. Such functional part for instance comprises a thyroid hormone binding site or at least a part of such a site, which is often comprising one or more peptides within the large polypeptide. In one embodiment said protein comprises rat monocarboxylate transporter-8 protein. Expressing recombinant protein and

isolating and purifying a protein or fragment thereof is a skill available in the art. For instance, a vector can be provided with a nucleic acid sequence encoding a monocarboxylate transporter-8 protein or a functional part, derivative and/or analogue thereof. Said vector can be administered to a host cell, for instance by a gene delivery vehicle. If said cell is capable of expressing said MCT protein, said MCT protein can be produced and isolated by methods known in the art.

A vector comprising a nucleic acid encoding a monocarboxylate transporter protein, preferably MCT-8, or a functional part, derivative and/or analogue thereof is also suitable for gene therapy. The same is true for a vector comprising a nucleic acid sequence encoding a molecule capable of specifically binding a monocarboxylate transporter protein, preferably MCT-8, or a functional part, derivative and/or analogue thereof, or for a vector capable of specifically binding a ligand of said monocarboxylate transporter protein. Such vectors are therefore also herewith provided.

The invention also provides a gene delivery vehicle comprising a vector of the invention. Methods for generating a gene delivery vehicle comprising a certain nucleic acid of interest are known in the art. A gene delivery vehicle of the invention is very suitable for treatment of a disorder such as a disorder of thyroid metabolism, non-thyroidal illness, obesity or cardiovascular illness with gene therapy.

Now that the invention provides the knowledge that monocarboxylate transporter protein is involved in thyroid hormone transport, it has become possible to investigate whether an individual suffers from, or is at risk of suffering from, disorders of thyroid hormone metabolism such as for instance brain disorders, tissue metabolism disorders, non-thyroidal illness, obesity and/or cardiovascular illness. It can be estimated whether thyroid hormone transport is adversely effected. This can for instance be done by determining whether an individual comprises a mutation in a nucleic acid of its genome

encoding a monocarboxylate transporter protein or a functional part, derivative and/or analogue thereof, or a ligand of said monocarboxylate transporter protein. A mutation in said nucleic acid, such as for instance a substitution, deletion or addition of at least one nucleotide, is indicative for impaired expression of said monocarboxylate transporter protein or a functional part, derivative and/or analogue thereof, or a ligand of said monocarboxylate transporter protein, resulting in impaired thyroid hormone transport.

This is confirmed by an investigation of a patient suffering from a thyroid hormone disorder characterized by high serum T3 and TSH titers, low T4 and rT3 titers, and mental disorder. Analysis of the MCT-8 gene of said patient has revealed that said gene lacks exon 1, while exons 2-6 are present. This shows that a mutation of a monocarboxylate transporter protein gene is involved with a thyroid hormone disorder. Analysis of said monocarboxylate transporter protein gene, or a gene of a ligand thereof, therefore provides an important diagnostic tool for detecting thyroid hormone disorders. Methods for detecting a mutation in a nucleic acid are known in the art. For instance, total nucleic acid of a sample may be isolated using a known method in the art and the nucleic acid may subsequently be amplified, preferably using specific primers for a gene encoding at least one monocarboxylate transporter protein or a ligand thereof. Subsequently, mutations in said gene can be detected, for instance using specific probes, by sequencing amplified product, etcetera.

The invention therefore provides a method for determining whether an individual is suffering from, or is at risk of suffering from, a thyroid hormone related disorder, non-thyroidal illness, obesity and/or cardiovascular illness, comprising determining whether said individual comprises a mutation in a nucleic acid sequence encoding a monocarboxylate transporter protein or a functional part, derivative and/or analogue thereof, and/or encoding a ligand of said monocarboxylate transporter protein or functional part, derivative and/or

analogue. In a preferred embodiment said mutation comprises a deletion. In a more preferred embodiment said mutation comprises a deletion of at least one exon.

In a further preferred embodiment a method of the invention is provided wherein said monocarboxylate transporter protein comprises monocarboxylate transporter protein-8.

A diagnostic kit comprising suitable means for detecting a mutation in a nucleic acid sequence encoding a monocarboxylate transporter protein or a functional part, derivative and/or analogue thereof, and/or encoding a ligand of said monocarboxylate transporter protein or functional part, derivative and/or analogue is also herewith provided.

The invention is further explained in the following examples. The examples do not limit the scope of the invention; they merely serve to exemplify the invention

Examples

Example 1. Transport of iodothyronines by MCT8 in oocytes

Xenopus oocytes were isolated and injected with 4.6 ng rat MCT8 cRNA. After 3 days, groups of 10 injected or uninjected oocytes were incubated for 60 min at 25 C with 10 nM radioactive iodothyronines in 0.1 ml medium. Uptake of iodothyronines was determined as previously described (18). The results are shown in figure 1.

Example 2 Transport of amino acids by MCT8 in oocytes

Xenopus oocytes were isolated and injected with cRNA coding for rat MCT8 or with cRNAs coding for the heavy chain (4F2) and the light chain (LAT1) of the heterodimeric human L-type amino acid transporter. After 3 days, groups of 10 injected or uninjected oocytes were incubated for 60 min at 25 C with 10 μM radioactive Leu, Tyr, Trp or Phe in 0.1 ml medium. Amino acid uptake was determined as previously described (18). The results are shown in figure 2.

Example 3. Saturation of T4 transport by MCT8 in oocytes

Xenopus oocytes were isolated and injected with rat MCT8 cRNA. After 3 days, groups of 10 injected or uninjected oocytes were incubated for 60 min at 25 C with radioactive T4 and increasing concentrations of nonradioactive T4 in 0.1 ml medium. T4 uptake was determined as previously described (18). The results are shown in figure 3.

Example 4. Time course of T3 uptake by MCT8 in oocytes

Xenopus oocytes were isolated and injected with rat MCT8 cRNA. After 3 days, groups of 10 injected or uninjected oocytes were incubated for 5-60 min at 25 C with 10 nM radioactive T3 in 0.1 ml medium. T3 uptake was determined as previously described (18). The results are shown in figure 4.

PROCEDURES

Materials - Nonradioactive L-iodothyronines, 3,3',5-triiodothyroacetic acid (Triac) and *N*-bromoacetyl-3,3',5-triiodothyronine ($BrAcT_3$) were obtained from Henning Berlin. D-iodothyronines, Phe and Tyr were purchased from Sigma, Leu was obtained from Merck, and Trp and bromosulfophthalein (BSP) were purchased from Fluka. [3',5'-^{125}I]T_4, [3'-^{125}I]T_3 and carrier-free Na^{125}I were purchased from Amersham Biosciences. All other ^{125}I-labeled compounds were prepared as previously described (21). ^{3}H-labeled Leu, Phe, Tyr, and Trp were purchased from Amersham Biosciences. All other chemicals were of reagent grade.

Cloning of rat MCT8 - Primers for RT-PCR were designed to regions of high homology in the 5' and 3' untranslated region (UTR) sequences of human (U05315) and mouse (AF045692) MCT8. The sense primer was 5' AGCTCTCGAGCGGCAAGCCACAGTCAG 3' corresponding to the mouse sequence from nucleotide (nt) 145 (the coding sequence starts at nt 175) and contained a *Xho*I restriction site (underlined). The antisense primer was 5' AAATGCGGCCGCTTCTCCGTTGGGGTCT 3' corresponding to the mouse sequence ending at nt 2242 (the coding sequence ends at nt 1872) and contained a *Not*I restriction site (underlined). Isolated rat liver mRNA was *in vitro* reverse transcribed and amplified using the reverse transcription system from Promega and subjected to PCR using the above primers. The 2038 bp product was ligated into pGEMT-Easy and sequenced.

Insertion of a FLAG-tagged MCT8 construct into the oocyte expression vector pGEM-HeJuel - For expression in oocytes it was decided to append an 8 amino acid "FLAG" epitope to the C-terminus of MCT8 in order to allow detection of expression by immunofluorescence microscopy and Western blotting. For this purpose the stop codon of MCT8 was removed by performing PCR using the

same sense primer as above, but with the modified antisense primer, 5' ACAGCGGCCGCAAATGGGCTCTTCAGGTGTTG 3' which lacks the stop codon. This PCR product was ligated into pGEMT-Easy before being excised with *Eco*RI and ligated into the FLAG vector, pCMV-Tag4A (Stratagene), which had previously been digested with the same restriction enzyme and dephosphorylated. The FLAG epitope-tagged MCT8 was then ligated into the *Xenopus* oocyte expression vector pGEM-HeJuel which contains the 5'- and 3'-UTRs of the *Xenopus* ß-globin flanking a multiple cloning site. The stability of the transcribed MCT8 mRNA sandwiched between the untranslated globin sequence is likely to be enhanced in the oocyte as was found to be the case for other MCTs (22). The MCT8-FLAG insert was prepared using PCR with primers flanking the MCT8-FLAG insert in pCMV-Tag4A and containing suitable restriction sites for the insertion into the pGEM-HeJuel vector. The sense primer was 5' GCGGGGATCCACACGTCAGTCCCCTAGCCA 3' and contained a *Bam*HI restriction site (underlined) whilst the antisense primer was 5' CTTATCTAGATAAGGTACCGGGCCCTACT 3' and contained a *Xba*I restriction site (underlined). Following successful PCR amplification, the product was digested with *Bam*HI and *Xba*I and ligated into the pGEM-HeJuel vector digested with the same restriction enzymes. The correct identity of the product was established using an *Eco*RI digest and confirmed with sequencing.

X. laevis oocyte expression - MCT8 cRNA was obtained by *in vitro* transcription using the Ampliscribe High Yield T7 RNA transcription kit (Epicentre) after linearization of pGEM-HeJuel containing the MCT8 cDNA with *Not*I. Oocytes were isolated and allowed to recover overnight at 18 C in modified Barth's solution containing 20 IU/ml penicillin and 20 µg/ml streptomycin as described before (23). The next day, oocytes were injected with cRNA coding for MCT8, and further incubated for 3 days at 18 C in modified Barth's solution until analysis. Uninjected or water injected oocytes were used as controls.

Western blotting of oocyte membranes - Crude oocyte membranes were prepared using 10 oocytes harvested 3 days after microinjection with MCT8 or MCT1 cRNA or the equivalent volume of water. Oocytes were suspended in 500 µl of buffer (10 mM HEPES, 83 mM NaCl, 1 mM $MgCl_2$, pH 7.9, containing 4 mg/ml pepstatin A, leupeptin, antipain and 0.5 mM phenylmethylsulfonyl fluoride and benzamidine) and homogenized by vigorous vortex mixing followed by several passes through an 18 gauge needle. The homogenate was centrifuged at 2000 rpm for 5 min at 4 C and the supernatant was collected, whilst the pellet was rehomogenized in 500 µl buffer and centrifuged as before. Both the supernatants were then mixed and centrifuged at 100,000 *g* for 60 min at 4 C and the pellet resuspended in 30 µl buffer and 30 µl of SDS-PAGE sample buffer. Following separation by SDS-PAGE Western blotting was performed using mouse anti-FLAG monoclonal antibody (Sigma) or the specific MCT1 antibody with detection by enhanced chemiluminescence (ECL) as described previously (24).

Preparation and staining of oocytes for immunofluorescence confocal microscopy - Oocytes were first embedded in pieces of chicken liver and then placed on pieces of cork, covered in O.C.T. embedding compound (Tissue-Tek, Sakura Finetek Europe B.V., The Netherlands) and frozen in liquid-nitrogen-cooled isopentane. Frozen sections (5 µm) were cut, placed on silanized slides and air-dried at room temperature for 1 h before fixing with ice-cold acetone for 10 min. Permeabilization and staining were then carried out as previously described (24) using a mouse monoclonal anti-FLAG antibody and TRITC-conjugated anti-mouse IgG secondary antibody. Samples were mounted with Mowiol (Calbiochem) and examined with a Leica TCS-NT confocal scanning microscope (63 x 1.32NA oil immersion objective).

Transport measurements in oocytes - Oocytes were washed and preincubated at 18 C in standard uptake solution (100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM Tris, pH 7.5). Usually, transport was tested by incubation of groups of 5-10 oocytes for 2-60 min at 25 C with 10 nM [^{125}I]iodothyronines or 10 μM [^{3}H]amino acids in 0.1 ml standard uptake solution. The possible Na^+ dependence of transport was tested by preincubation and incubation in Na^+-free uptake solution in which NaCl was replaced by choline chloride. The influence of the temperature on the uptake rate was tested by the incubation of oocytes with uptake solutions at different temperatures between 4 and 37 C. The influence of albumin on uptake of T_3 was tested by the addition of 0.1% and 0.5% BSA to the standard uptake solution. The incubation was stopped by aspiration of the medium, and oocytes were washed 4 times at 4 C with standard uptake solution containing 0.1% BSA.

The substrate specificity of MCT8 was investigated by incubation of oocytes with different putative radioactive ligands, including T_4, T_3, 3,3',5'-triiodothyronine (rT_3), 3,3'-diiodothyronine (T_2), *N*α-sulfonated T_4 (T_4 sulfamate, T_4NS) and 4'-OH-sulfonated T_4 (T_4 sulfate, T_4S), and the amino acids Leu, Phe, Tyr and Trp. Specificity of transport of ^{125}I-T_4 and ^{125}I-T_3 was measured in the presence of putative competitors, including 10 μM unlabeled iodothyronine derivatives such as D- and L-iodothyronines, Triac and $BrAcT_3$, and 100 μM Tyr, Trp or BSP.

Analysis of rT_3 metabolism in oocytes - Groups of 10 oocytes were incubated for 2-60 min at 25 C with 10 nM [^{125}I]rT_3. After incubation, medium was collected, and 2 groups of 5 oocytes were counted separately and homogenized in 0.1 ml 0.1 M NaOH as described before (25). Lysates were cleared by centrifugation. Lysates (in duplicate) and incubation media were acidified with 0.1 M HCl and analyzed by Sephadex LH-20 chromatography (21). The different products

were successively eluted with 0.1 M HCl (iodide), water (conjugates), and 1% NH_4OH in ethanol (iodothyronines).

Transport kinetics - Saturation of iodothyronine uptake in MCT8 cRNA-injected oocytes was analyzed in incubations containing labeled and unlabeled T_4, T_3, or rT_3 at final concentrations of 1 nM-30 μM. Apparent K_m values were calculated by fitting the plot of uptake rate (v) versus ligand concentration (S) to the Michaelis Menten equation: $v=V_{max}/(1+K_m/S)$, where V_{max} is the maximum uptake rate, and K_m the Michaelis constant. Calculations were performed using the Slide Write Plus program version 5.01 (Advanced Graphics Software).

Statistics - Results are expressed as means ± SEM. Statistical significance was determined using the Student's *t* test for unpaired observations.

RESULTS

Cloning of rat MCT8 - The coding sequence of rat MCT8 was cloned using RT-PCR from rat liver mRNA as described in the experimental section and has been asigned the accession code NM_147216 (gi:22219453). The translated protein sequence is shown in Fig. 5 where it is aligned with human (U05315) and mouse (AF045692) MCT8 sequences. As would be predicted, the rat and mouse sequences show very few differences with only four amino acids changes and the insertion of a 20 amino acid repeat in the mouse PEST sequence which is absent in the rat and human sequences. The predicted molecular mass of the protein is 60.1 kDa.

Expression of the FLAG-tagged MCT8 in Xenopus oocytes - We initially sought to express the MCT8 in oocytes but the antibody we raised to the C-terminus of the protein (which we have used successfully for production of antibodies against other MCTs) failed to detect native and recombinant rat MCT8. Thus, we expressed MCT8 with a FLAG epitope attached to the C-terminus as described in the experimental section. The cloning strategy used means that the C-terminus is extended by the following sequence (FLAG-tag underlined) CAAVITSEFDIKLIDTVDLE<u>DYKDDDDK</u> giving a predicted molecular mass 63.3 kDa.

In Fig. 6 we present Western blots using an anti-FLAG antibody of membranes derived from oocytes injected with water (control), MCT1 (26).or MCT8-FLAG cRNA. A band of about 63 kDa is present only in the membranes from the MCT8 cRNA-injected oocytes (lanes 3 and 4) confirming the presence of the expressed FLAG-tagged MCT8. The band at 38 kDa is a FLAG-sensitive band in *X. laevis* oocytes. Plasma membranes from oocytes injected with MCT1 cRNA showed no response to the FLAG antibody but did show a 43 kDa protein corresponding to MCT1 with the specific MCT1 antibody (lane 2, lower

part). The latter failed to detect the MCT8 as would be expected in view of the lack of similarity between the C-termini of MCT8 and MCT1.

In order to confirm that MCT8 was expressed at the plasma membrane we performed immunofluorescence microscopy on sections of oocytes, again using the anti-FLAG antibody. The data are shown in Fig. 7 and reveal that MCT8 is strongly expressed at the plasma membrane. Water-injected oocytes showed no such expression; nor did the secondary antibody alone detect any protein at the plasma membrane.

Functional characterization - Transport studies were performed to investigate the function of MCT8 expressed in *X. laevis* oocytes. Figure 8 shows the time course of uptake of T_3 and T_4 in uninjected oocytes and in oocytes injected with 4.6 ng MCT8 cRNA. Expression of MCT8 induced a ~10-fold increase in initial uptake of T_3 and T_4 compared with uninjected oocytes. This graph also shows that the uptake of T_3 and T_4 into MCT8 cRNA-injected oocytes was only linear for the first 4 min. Therefore, all further transport experiments were performed at 2 min incubations. The difference in uptake of T_3 and T_4 in oocytes injected with MCT8 cRNA is not statistically significant. Transport of T_3 was independent of Na^+ as the same results were obtained using medium with choline chloride instead of NaCl (Fig. 8A). However, T_4 transport by MCT8 showed a modest but consistent inhibition in the absence of Na^+ (Fig. 8B).

Figure 9 shows the influence of the amount of cRNA injected on the uptake of 10 nM T_3 and T_4. The results indicate that the lowest amount of MCT8 cRNA (0.23 ng) injected already induced a 5-fold increase in T_3 transport and a 7.3-fold increase in T_4 transport. Maximum induction of T_3 and T_4 transport was found after injection of 1.15 - 2.3 ng of MCT8 cRNA. Therefore, oocytes were further injected with 4.6 ng MCT8 cRNA for maximum induction of iodothyronine transport.

To test the temperature dependence of MCT8-mediated iodothyronine transport, oocytes were incubated with 10 nM [^{125}I]T_3 or [^{125}I]T_4 for 2 min at 4-37 C. Figure 10 shows significant uptake for T_3 and T_4 into the oocytes at 4 C, with marked increases if the temperature was increased to 15, 25 and 37 C, which is above the usual ambient temperature for frog oocytes. Exposure to 37 C was tolerated by the oocytes during this short incubation time; prolonged incubation at 37 C resulted in desintegration of the cells. The results show identical temperature dependence of transport of T_4 and T_3 by MCT8.

Figure 11 shows the effects of addition of 0.1% and 0.5% BSA on the uptake of T_3 in oocytes. Both in uninjected oocytes and in MCT8 cRNA-injected oocytes, T_3 uptake was decreased concentration-dependently by BSA. However, the fold stimulation of T_3 uptake by MCT8 increased from 7.5 in the absence of BSA to 14 in the presence of 0.1% BSA, and further to 25 in the presence of 0.5% BSA.

Substrate specificity - The substrate specificity of MCT8 was investigated by incubation of oocytes with different putative radioactive ligands, including T_4, T_3, rT_3, T_2, T_4NS and T_4S, and the amino acids Leu, Phe, Tyr and Trp. Figure 12 shows that in contrast to the rapid uptake of the different iodothyronines, T_4NS and T_4S and the amino acids are not transported by MCT8. MCT8 failed to transport the different amino acids at concentrations (1-100 μM) showing facile transport by the LAT1 (18) and TAT1 (19, 20) amino acid transporters. We have also been unable to demonstrate any transport of [^{14}C]lactate, whereas in oocytes injected with rat MCT1 cRNA, transport of [^{14}C]lactate was greatly stimulated compared to control oocytes, but no additional uptake of [^{125}I]T_4 was observed (data not shown).

The specificity of iodothyronine transport by MCT8 was further addressed by investigating the uptake of [^{125}I]T_3 and [^{125}I]T_4 in the absence or presence of structurally related compounds (Table 1). Uptake of labeled T_4 and T_3 by MCT8 was potently inhibited by 10 μM unlabeled L-T_4, D-T_4, L-T_3 and D-

T_3, indicating that the interaction of T_4 and T_3 with MCT8 is not stereospecific. Iodothyronine uptake by MCT8 is also potently inhibited by T_3 analogs where the αNH_2 group is blocked ($BrAcT_3$) or deleted (Triac), indicating that this αNH_2 group is not important for interaction of iodothyronines with MCT8. The T-type amino acids Tyr and Trp hardly affected iodothyronine uptake by MCT8. The organic anion BSP proved to be a potent inhibitor. This in contrast to the weak effects of the bile acid taurocholate and the organic anion transport inhibitor probenecid (data not shown). In general, T_3 transport by MCT8 shows less inhibition by these compounds than MCT8-mediated T_4 transport. It is remarkable that ligands and potent inhibitors of MCT8 all carry halogen atoms.

Metabolism of rT_3 - Previous studies have shown that *X. laevis* oocytes actively metabolize rT_3 by sulfation. Since sulfotransferases are located intracellularly, we have used this property to investigate rT_3 internalization by oocytes. Figure 13 shows the time course of rT_3 uptake and subsequent metabolism in native and MCT8-expressing oocytes. At several time points, uninjected and MCT8 cRNA-injected oocytes are homogenized, and the homogenates and medium samples are analyzed for rT_3 and rT_3 sulfate (rT_3S) content. It is shown that already after 2 min of incubation, rT_3S is formed intracellularly in the MCT8 cRNA-injected oocytes. After 10 min of incubation, release of rT_3S into the medium is observed. The production rate of rT_3S is much higher in MCT8 cRNA-injected oocytes than in uninjected cells. These findings demonstrate that MCT8-mediated uptake of iodothyronines indeed represents the internalization of these compounds.

Transport kinetics - The kinetics of MCT8-mediated iodothyronine transport were analyzed by incubation of oocytes with increasing T_4, T_3 or rT_3 concentrations (1 nM – 30 μM). Net transport by MCT8 was calculated by subtracting uptake in uninjected oocytes from that in MCT8 cRNA-injected

oocytes. The results showed that transport of T_4, T_3 and rT_3 by MCT8 was saturable. Michaelis-Menten analysis of the data provided apparent K_m values of 4.7 μM for T_4, 4.0 μM for T_3 and 2.2 μM for rT_3 (Fig. 14).

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Brief description of the drawings

Figure 1: Transport of iodothyronines by MCT8 in oocytes

Figure 2: Transport of amino acids by MCT8 in oocytes

Figure 3: Saturation of T4 transport by MCT8 in oocytes

Figure 4: Time course of T3 uptake by MCT8 in oocytes

Figure 5: Amino sequence alignment of rat, mouse and human MCT8. Alignment was performed using the ClustalW program (DNASTAR LaserGene software). Identity between 3 species are indicated in black, and identity between 2 species in gray.

Figure 6: Western blot to show expression of FLAG-tagged MCT8 in *X. laevis* oocytes. Plasma membrane preparations were prepared from oocytes 3 days after injection with water (lane 1), MCT1 (lane 2) or MCT8-FLAG cRNA (lane 3 and 4) and subjected to SDS-PAGE and Western blotting with the anti-FLAG antibody or the anti-MCT1 antibody as described in the experimental section.

Figure 7: Detection of FLAG-tagged MCT8 protein at the cell surface of the oocytes. Oocytes were fixed, sectioned and prepared for immunofluorescence microscopy (anti-FLAG antibody) 3 days after injection with MCT8-FLAG cRNA or water (control) as described in the experimental section.

Figure 8: Time course of T_3 and T_4 uptake in oocytes. Oocytes were injected with MCT8 cRNA and after 3 days incubated for 2-60 min at 25 C with 10 nM

[^{125}I]T_3 (A) or [^{125}I]T_4 (B) in medium with or without Na^+. In the latter NaCl was replaced by choline chloride.

Figure 9: MCT8 cRNA concentration-dependent uptake of T_3 and T_4. Oocytes were injected with 0-11.5 ng MCT8 cRNA and after 3 days incubated for 2 min at 25 C with 10 nM T_3 (●) or T_4 (○).

Figure 10: Temperature-dependent uptake of T_3 and T_4 by MCT8. Oocytes were incubated for 2 min at 4, 15, 25 or 37 C with 10 nM T_3 (■) or T_4 (□). Net uptake of T_3 or T_4 in MCT8 cRNA-injected oocytes is corrected for the corresponding uptake in uninjected oocytes.

Figure 11: Albumin-dependent uptake of T_3. Oocytes were incubated for 2 min at 25 C in standard uptake solution with 10 nM T_3 without albumin, or supplemented with 0.1 % or 0.5 % albumin (■). Uninjected oocytes were used as controls (□). Numbers indicate fold stimulation by MCT8.

Figure 12: Ligand-dependent transport by MCT8. MCT8 cRNA-injected oocytes were incubated for 2-60 min at 25 C with 10 nM [^{125}I]iodothyronines or 10 μM [^{3}H]amino acids (Tyr, Trp, Leu and Phe) (■). Uninjected oocytes were used as controls (□). The uptake of the different putative ligands is expressed per min.

Figure 13: Uptake and metabolism of rT_3. Oocytes were incubated for 2-60 min at 25 C with 10 nM rT_3. At each time point, uninjected oocytes (left panel) and MCT8 cRNA-injected oocytes (right panel) were homogenized, and homogenates and medium samples were analyzed as is described in experimental procedures.

Figure 14: Kinetics of MCT8 mediated rT_3, T_3 and T_4 uptake. Oocytes were incubated with increasing rT_3, T_3 or T_4 concentrations (1 nM - 30 μM). Net transport by MCT8 was calculated by subtracting uptake in uninjected oocytes from that in MCT8 cRNA-injected oocytes. Uptake is expressed as fmol / oocyte * min. Kinetics were performed using the Slide Write Plus Program (Advanced Graphics Software).

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Claims

1. Use of a monocarboxylate transporter protein or a functional part, derivative and/or analogue thereof for altering transport of a thyroid hormone or a functional part, derivative and/or analogue thereof across a membrane.

2. An isolated molecule capable of specifically binding a monocarboxylate transporter protein or a functional part, derivative and/or analogue thereof, or a ligand of said monocarboxylate transporter protein.

3. An isolated molecule capable of specifically binding a nucleic acid sequence encoding a monocarboxylate transporter protein or a functional part, derivative and/or analogue thereof, and/or encoding a ligand of said monocarboxylate transporter protein or functional part, derivative and/or analogue thereof.

4. Use of a molecule according to claim 2 or 3 for altering transport of a thyroid hormone or a functional part, derivative and/or analogue thereof across a membrane.

5. Use according to claim 1 or 4, wherein said monocarboxylate transporter protein comprises monocarboxylate transporter protein-8.

6. Use according to claim 1, 4 or 5 wherein said membrane comprises a plasma membrane.

7. Use according to any one of claims 1 or 4-6, wherein said transport of said thyroid hormone or functional part, derivative and/or analogue thereof is enhanced.

8. A compound capable of influencing the binding or transporting of a ligand of, or capable of binding to, a plasma membrane polypeptide capable of transporting a thyroid hormone, wherein said polypeptide comprises a monocarboxylate transporter protein or a functional part, derivative and/or analogue thereof, for use as a medicament.

9. A compound according to claim 8 wherein said polypeptide is capable of transporting 3,3',5-triiodothyronine.

10. A compound according to claim 8 or 9 wherein said monocarboxylate transporter protein comprises monocarboxylate transporter protein-8.

11. Use of a compound according to anyone of claims 8-10 for the production of a medicament for the treatment of a thyroid hormone related disorder, non-thyroidal illness, obesity or cardiovascular illness.

12. A pharmaceutical composition comprising a compound according to anyone of claims 8-10, and a suitable carrier.

13. A method for altering transport of a thyroid hormone or a functional part, derivative and/or analogue thereof across a membrane comprising providing said membrane with a monocarboxylate transporter protein or a functional part, derivative and/or analogue thereof, and/or with a molecule capable of specifically binding a monocarboxylate transporter protein or a functional part, derivative and/or analogue thereof, or a ligand of said monocarboxylate transporter protein, and/or with a molecule capable of specifically binding a nucleic acid sequence encoding a monocarboxylate transporter protein or a functional part, derivative and/or analogue thereof, or encoding a ligand of said monocarboxylate transporter protein or functional part, derivative and/or analogue.

14. A method for treating a disorder such as a disorder of thyroid metabolism, non-thyroidal illness, obesity or cardiovascular illness, comprising administering a compound according to anyone of claims 8-10 or a pharmaceutical composition according to claim 12 to an individual suffering from said disorder.

15. Use of at least a functional part of a monocarboxylate transporter protein, or a nucleic acid encoding at least a functional part of a monocarboxylate transporter protein, in a method for treating a disorder such as a thyroid hormone related disorder such as a disorder of thyroid metabolism, non-thyroidal illness, obesity or cardiovascular illness.

16. Method according to claim 13 or 14 or use according to claim 15, comprising gene therapy.

17. Use of at least a functional part of a monocarboxylate transporter protein, or a nucleic acid encoding at least a functional part of a monocarboxylate transporter protein, in a method for detecting and/or generating a pharmaceutical compound.

18. A method according to claim 13,14 or 16 or a use according to claim 15 or 17 wherein said monocarboxylate transporter protein comprises monocarboxylate transporter protein-8.

19. A bioassay to identify or detect a candidate drug capable of binding to or influencing a plasma membrane polypeptide capable of transporting a thyroid hormone, wherein said polypeptide comprises a monocarboxylate transporter protein or a functional part, derivative and/or analogue thereof.

20. A bioassay according to claim 19 using at least a functional part of a monocarboxylate transporter protein, a nucleic acid encoding at least a functional part of a monocarboxylate transporter protein, a molecule capable of specifically binding at least part of said monocarboxylate transporter protein or a ligand thereof, a cell comprising said nucleic acid and/or a cell comprising at least part of said protein.

21. A bioassay according to claim 19 or 20 wherein said monocarboxylate transporter protein comprises monocarboxylate transporter protein-8.

22. An isolated monocarboxylate transporter-8 protein or a functional part, derivative and/or analogue thereof.

23. A protein according to claim 22, wherein said protein comprises rat monocarboxylate transporter-8 protein.

24. A vector comprising a nucleic acid sequence encoding a monocarboxylate transporter-8 protein or a functional part, derivative and/or analogue thereof, and/or a molecule capable of specifically binding a monocarboxylate transporter-8 protein or a functional part, derivative and/or analogue thereof,

or capable of specifically binding a ligand of said monocarboxylate transporter-8 protein.

25. A gene delivery vehicle comprising a vector according to claim 24.

26. A method for determining whether an individual is suffering from, or is at risk of suffering from, a thyroid hormone related disorder, non-thyroidal illness, obesity and/or cardiovascular illness, comprising determining whether said individual comprises a mutation in a nucleic acid sequence encoding a monocarboxylate transporter protein or a functional part, derivative and/or analogue thereof, and/or encoding a ligand of said monocarboxylate transporter protein or functional part, derivative and/or analogue.

27. A method according to claim 26, wherein said mutation comprises a deletion.

28. A method according to claim 26 or 27, wherein mutation comprises a deletion of at least one exon.

29. A method according to any one of claims 26-28, wherein said monocarboxylate transporter protein comprises monocarboxylate transporter protein-8.

30. A diagnostic kit comprising suitable means for detecting a mutation in a nucleic acid sequence encoding a monocarboxylate transporter protein or a functional part, derivative and/or analogue thereof, and/or encoding a ligand of said monocarboxylate transporter protein or functional part, derivative and/or analogue.

Table 1 Inhibition of uptake of T_4 and T_3 (10 nM) in MCT8 cRNA-injected oocytes by iodothyronines or Tyr, Trp and BSP.

		% inhibition±SEM	
Inhibitor	μM	T_4	T_3
L-T_4	10	69±7	26±7
L-T_3	10	62±7	57±3
D-T_4	10	76±7	21±5
D-T_3	10	72±3	24±5
Triac	10	76±3	47±3
$BrAcT_3$	10	90±3	67±1
Tyr	100	37±6	0±9
Trp	100	22±6	0±7
BSP	100	94±2	81±1

Figure 1

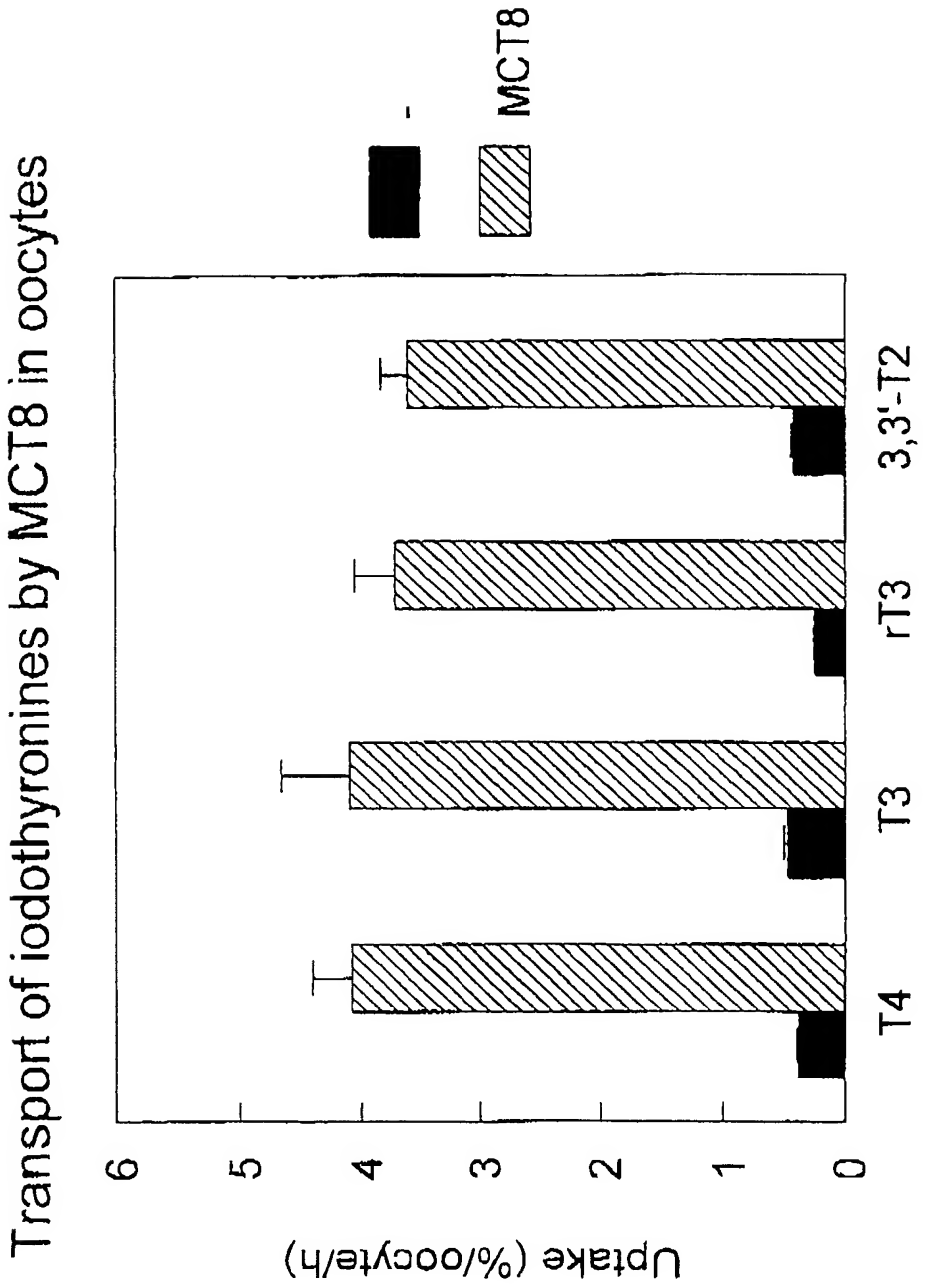

Transport of iodothyronines by MCT8 in oocytes
Uptake (%/oocyte/h)
0
1
2
3
4
5
6
T4
T3
rT3
3,3'-T2
-
MCT8

Figure 2

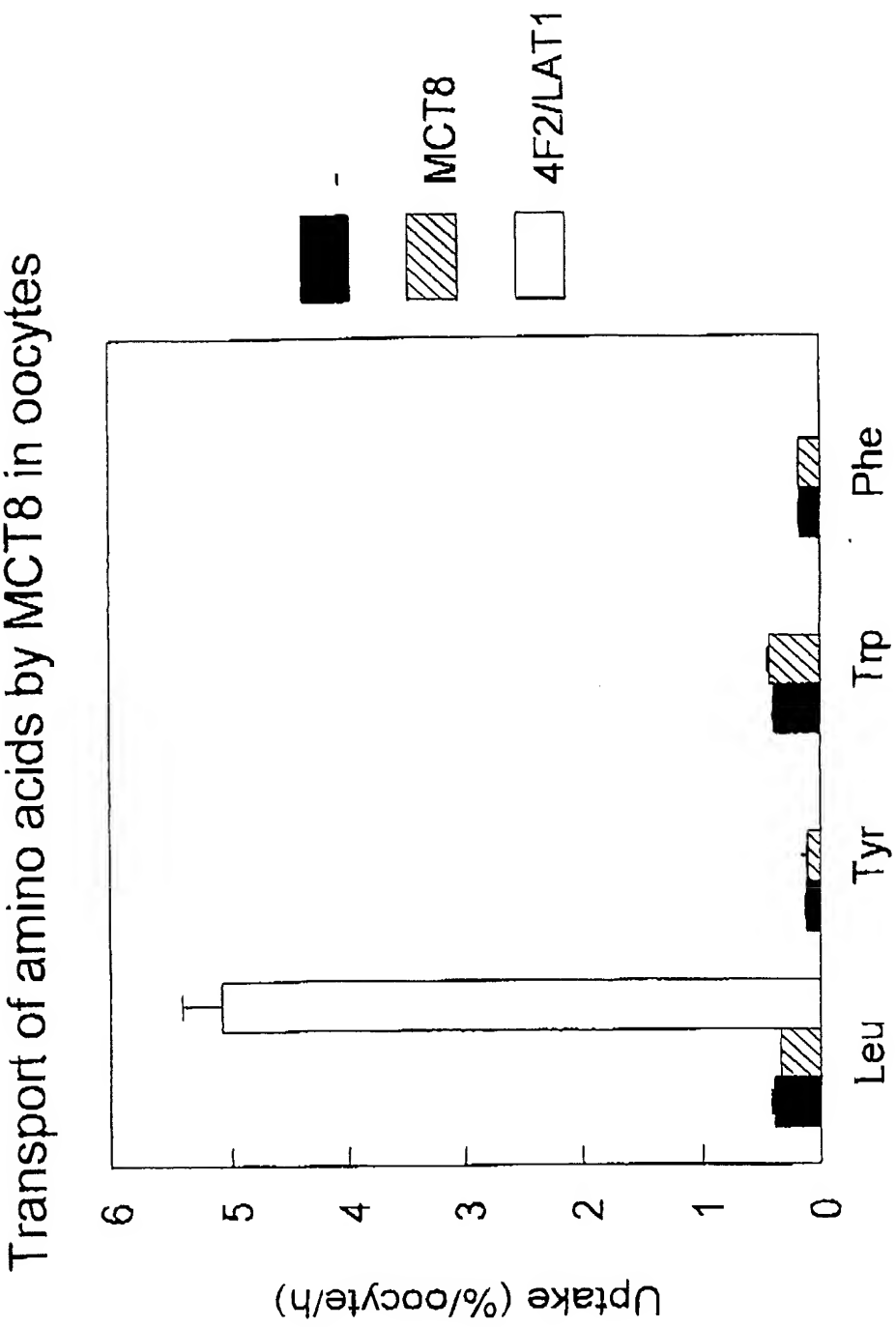

Transport of amino acids by MCT8 in oocytes
-
MCT8
4F2/LAT1
Uptake (%/oocyte/h)
0
1
2
3
4
5
6
Leu
Tyr
Trp
Phe

Figure 3

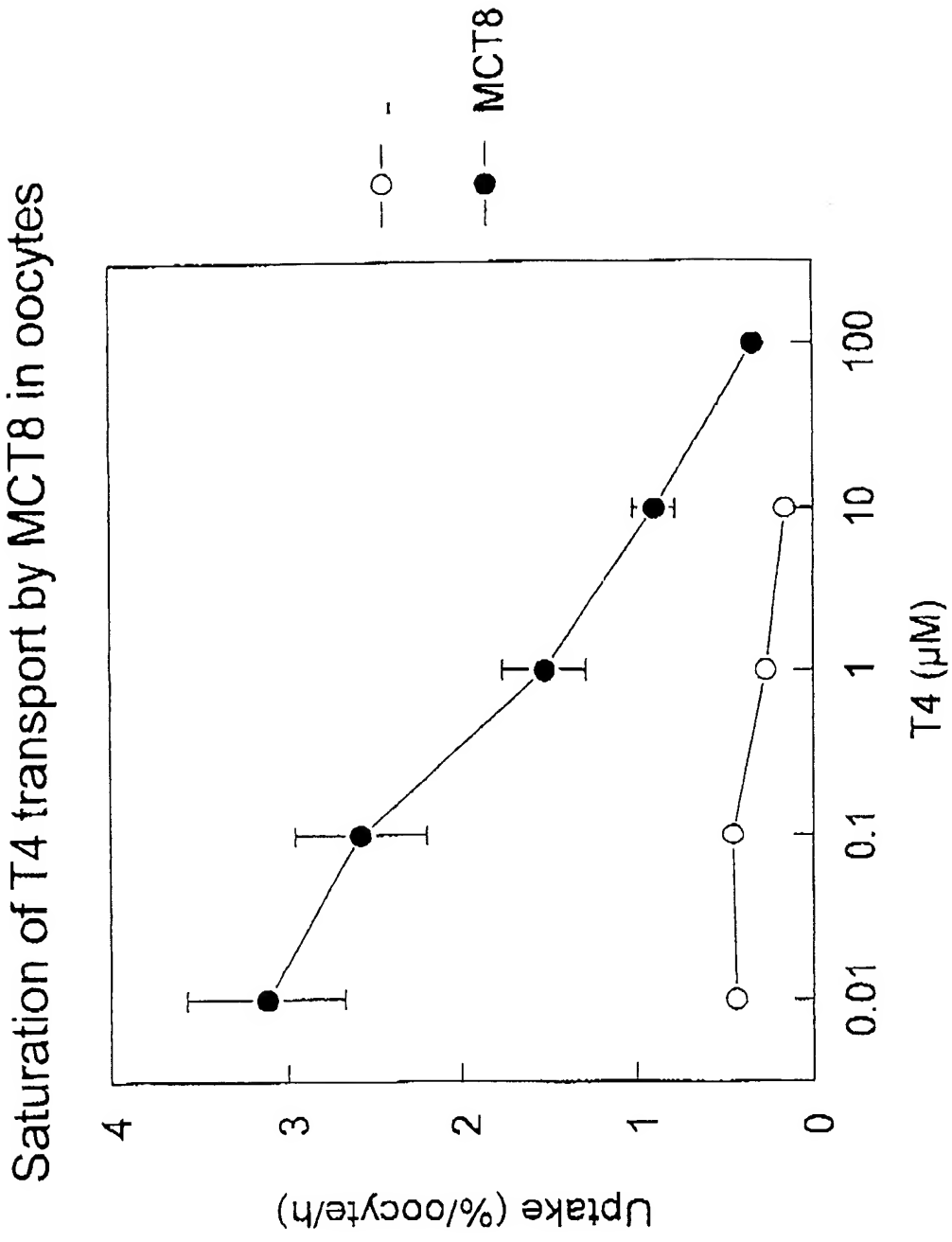

Saturation of T4 transport by MCT8 in oocytes
MCT8
-
Uptake (%/oocyte/h)
0
1
2
3
4
0.01
0.1
1
10
100
T4 (µM)

Figure 4

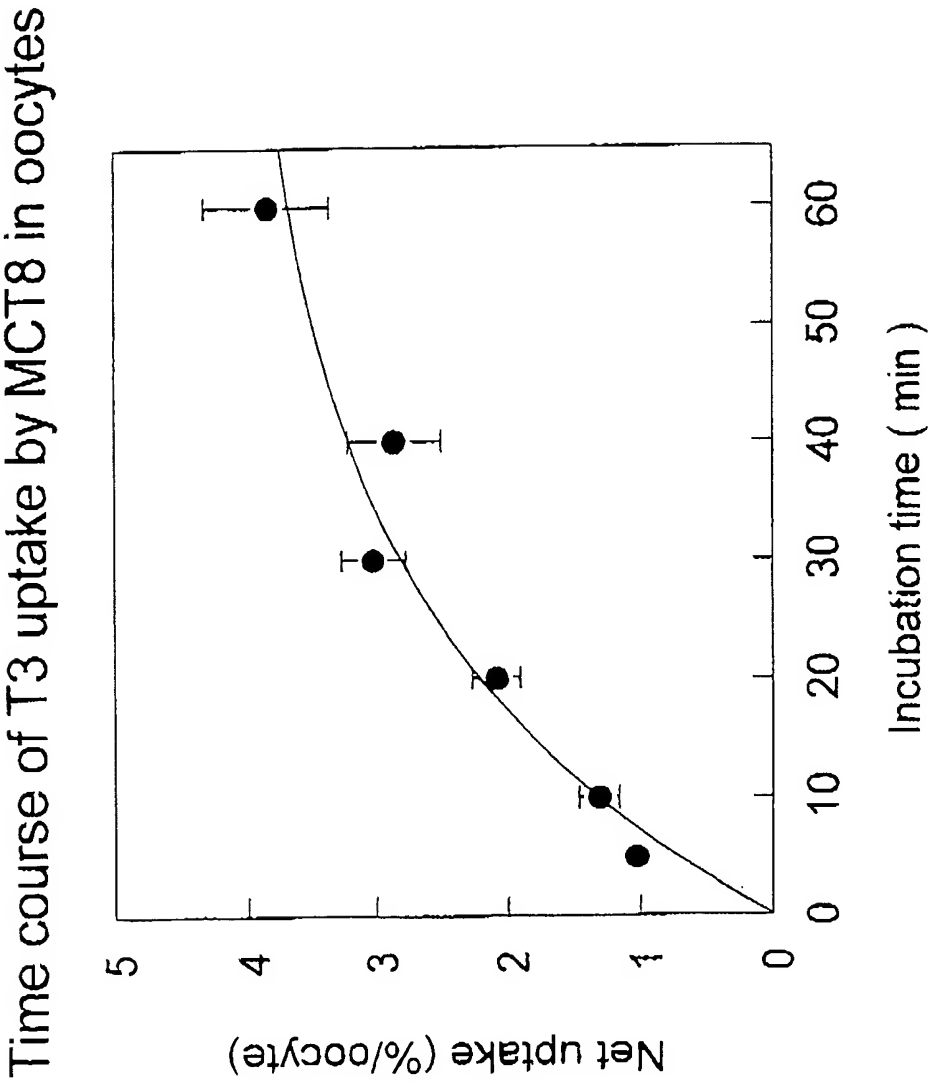

Time course of T3 uptake by MCT8 in oocytes
Net uptake (%/oocyte)
0
1
2
3
4
5
0
10
20
30
40
50
60
Incubation time (min)

Fig. 5

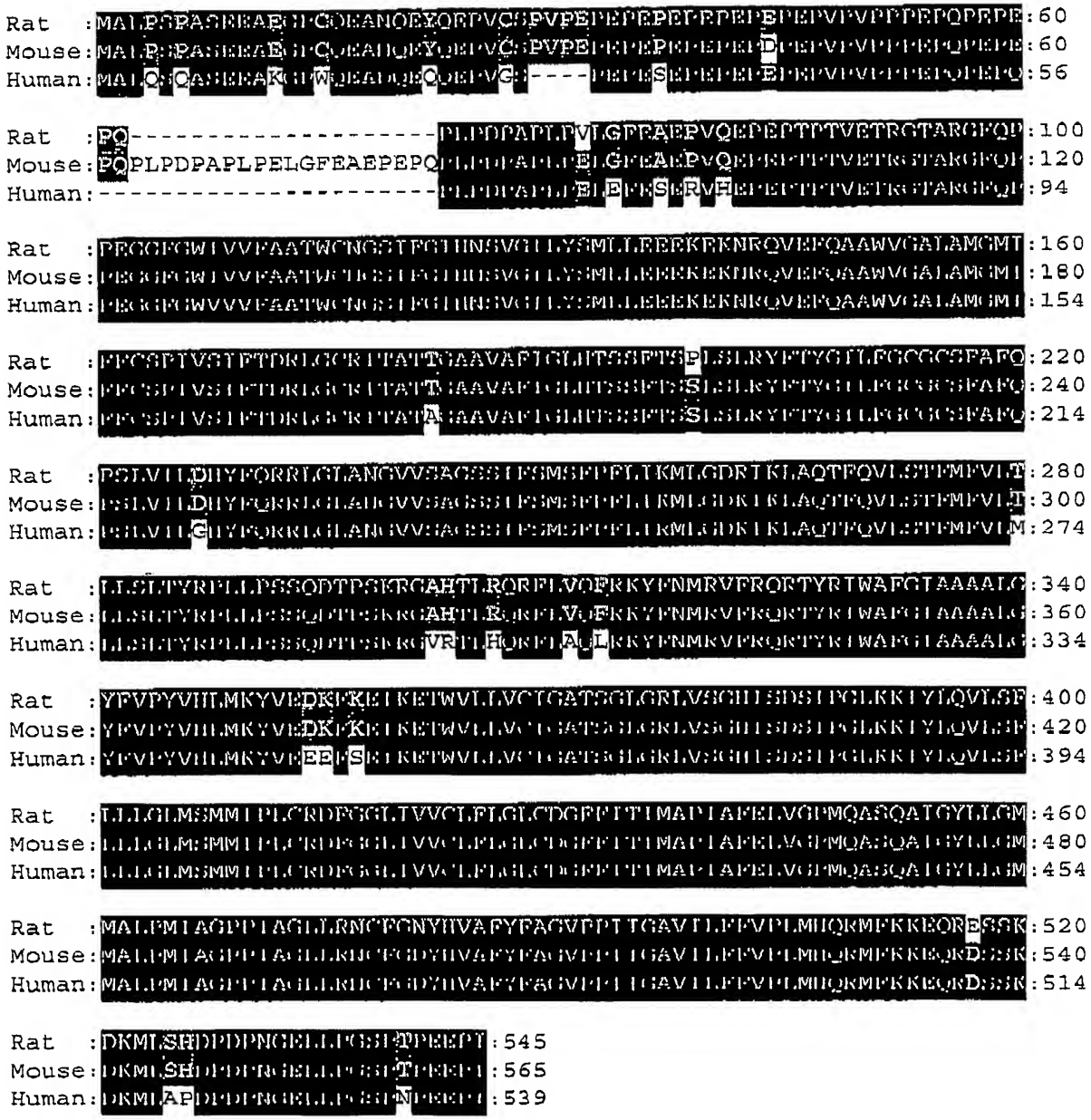


Fig. 6

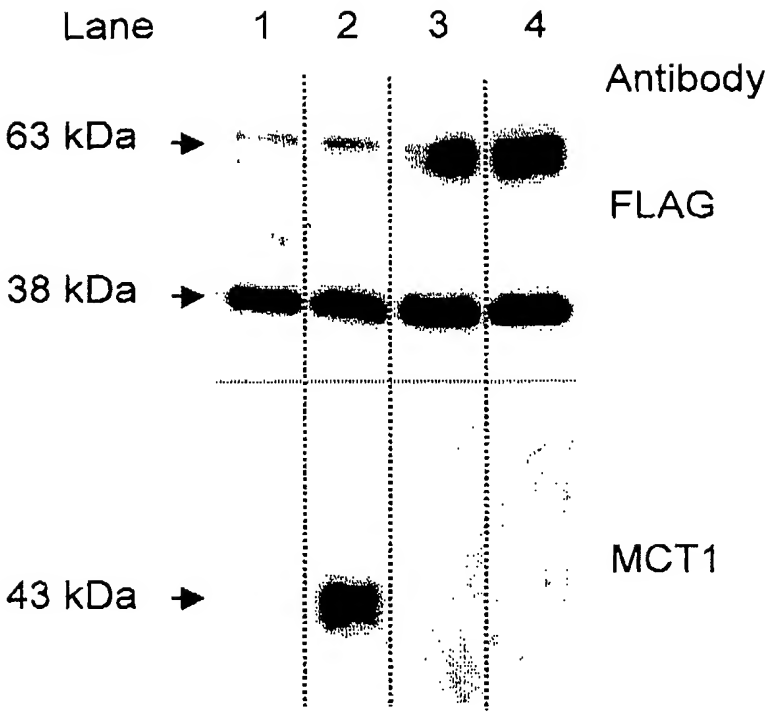


Fig. 7

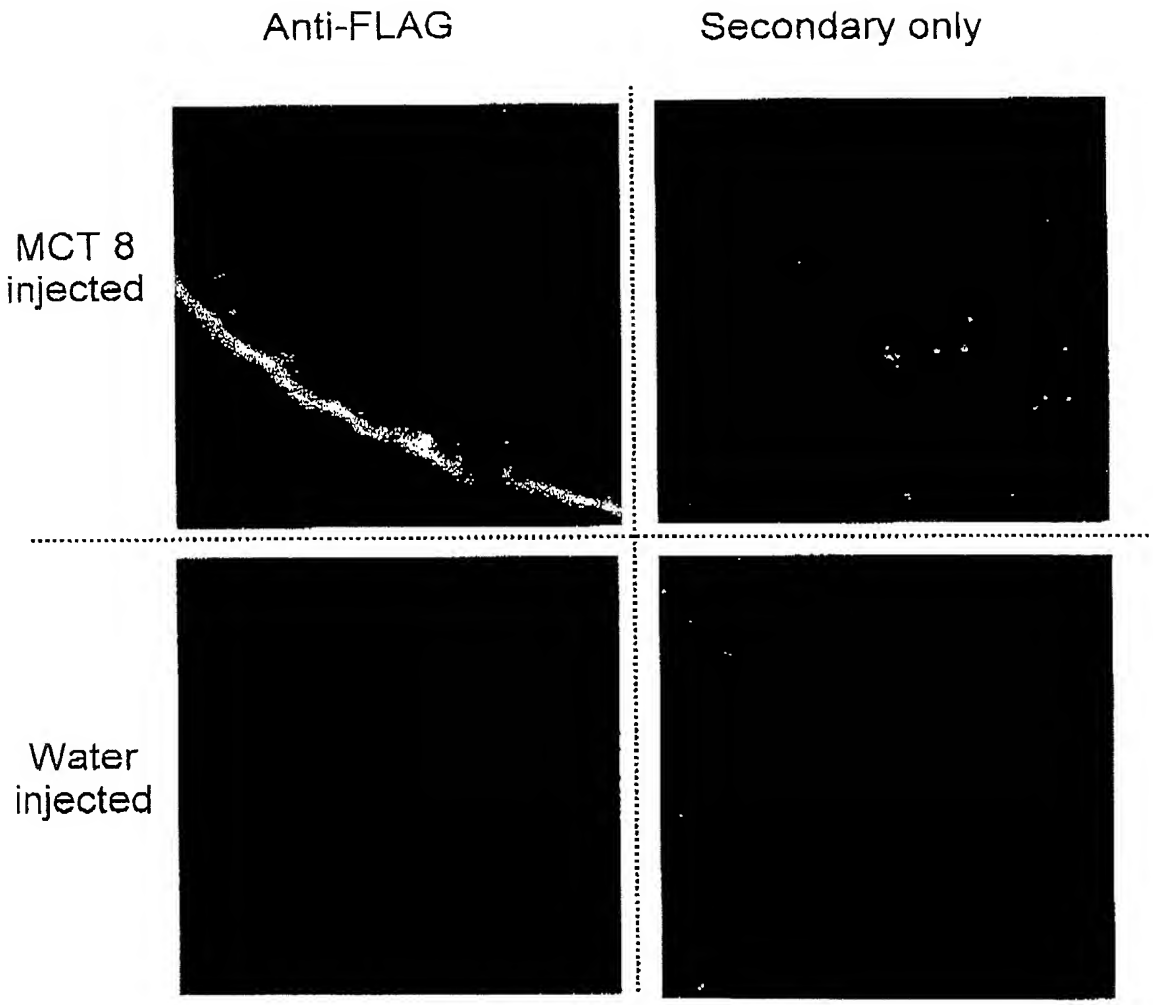


Fig. 8

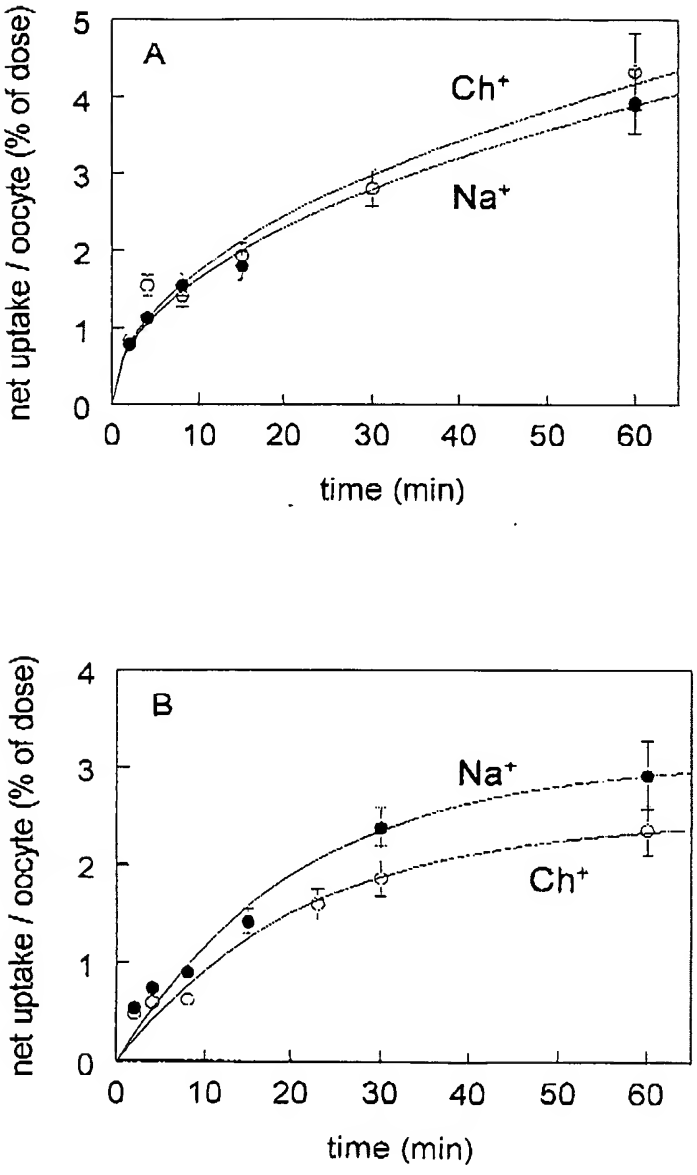

A
Ch+
Na+
net uptake / oocyte (% of dose)
time (min)
0
1
2
3
4
5
0
10
20
30
40
50
60
B
Na+
Ch+
net uptake / oocyte (% of dose)
time (min)
0
1
2
3
4
0
10
20
30
40
50
60

Fig. 9

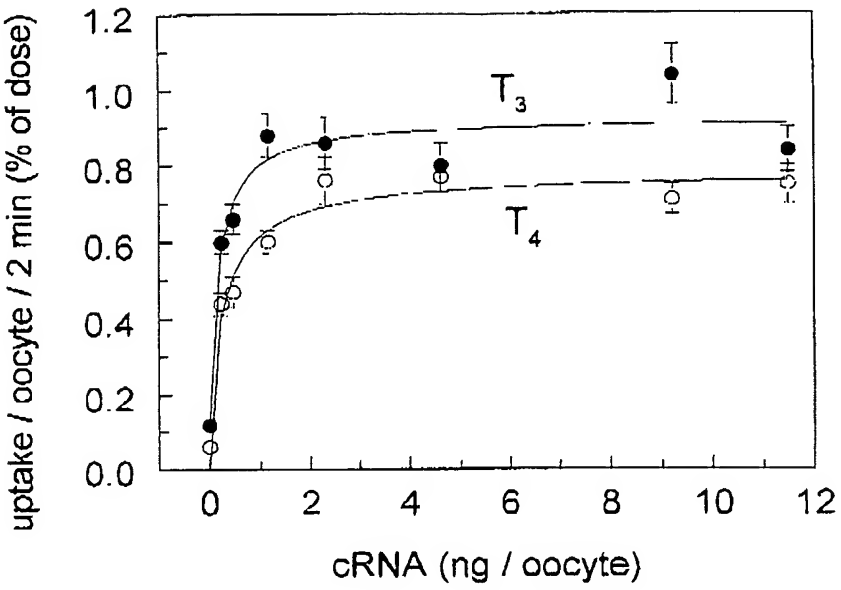

uptake / oocyte / 2 min (% of dose)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
12
cRNA (ng / oocyte)
T3
T4

Fig.10

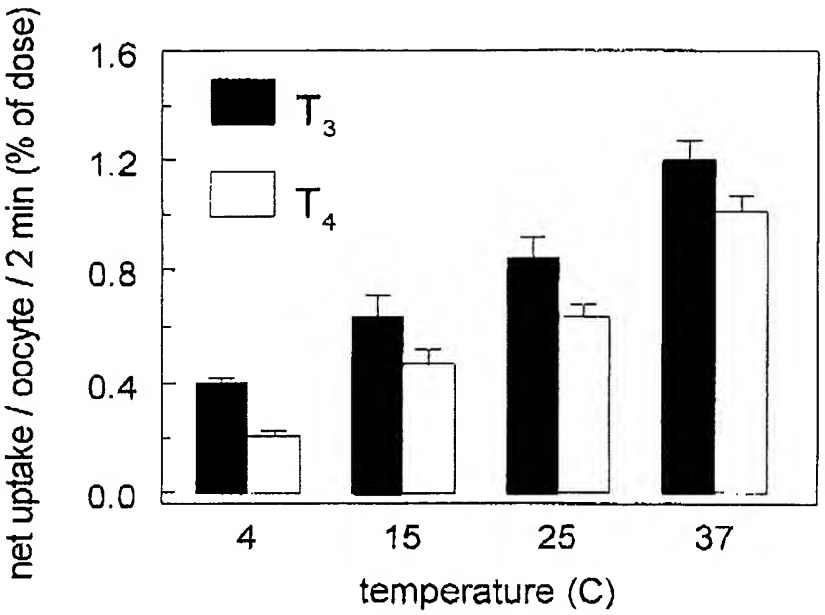

net uptake / oocyte / 2 min (% of dose)
1.6
1.2
0.8
0.4
0.0
T3
T4
4
15
25
37
temperature (C)

Fig. 11

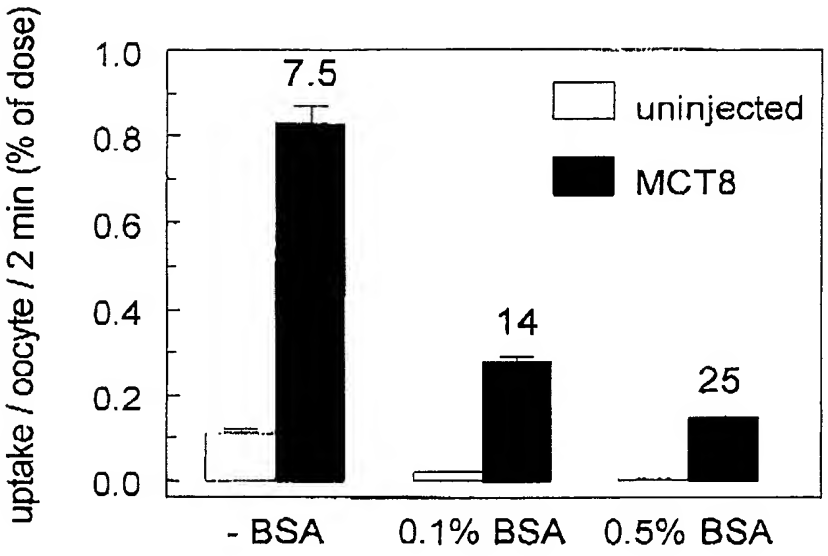

uptake / oocyte / 2 min (% of dose)
1.0
0.8
0.6
0.4
0.2
0.0
7.5
14
25
uninjected
MCT8
- BSA
0.1% BSA
0.5% BSA

Fig. 12

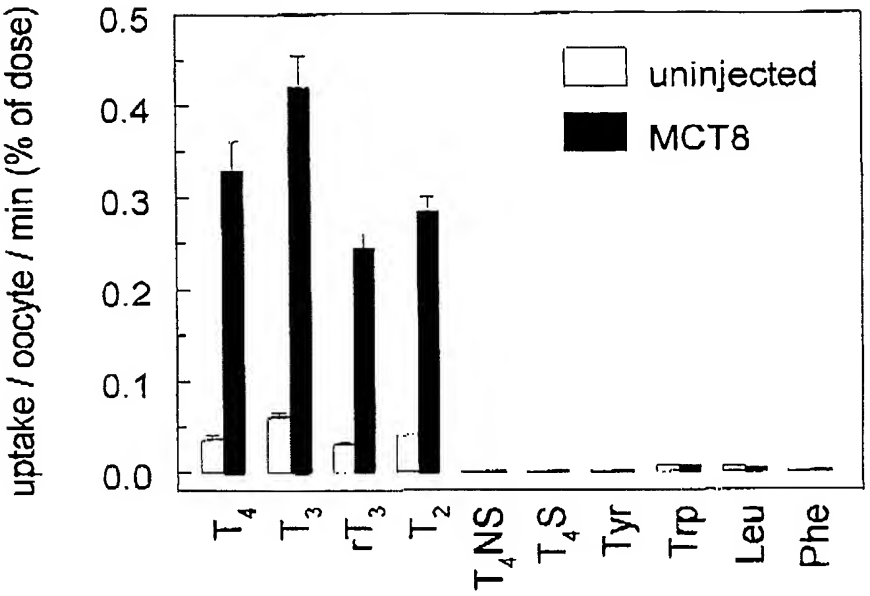

uptake / oocyte / min (% of dose)
0.5
0.4
0.3
0.2
0.1
0.0
uninjected
MCT8
T4
T3
rT3
T2
T4NS
T4S
Tyr
Trp
Leu
Phe

Fig. 13

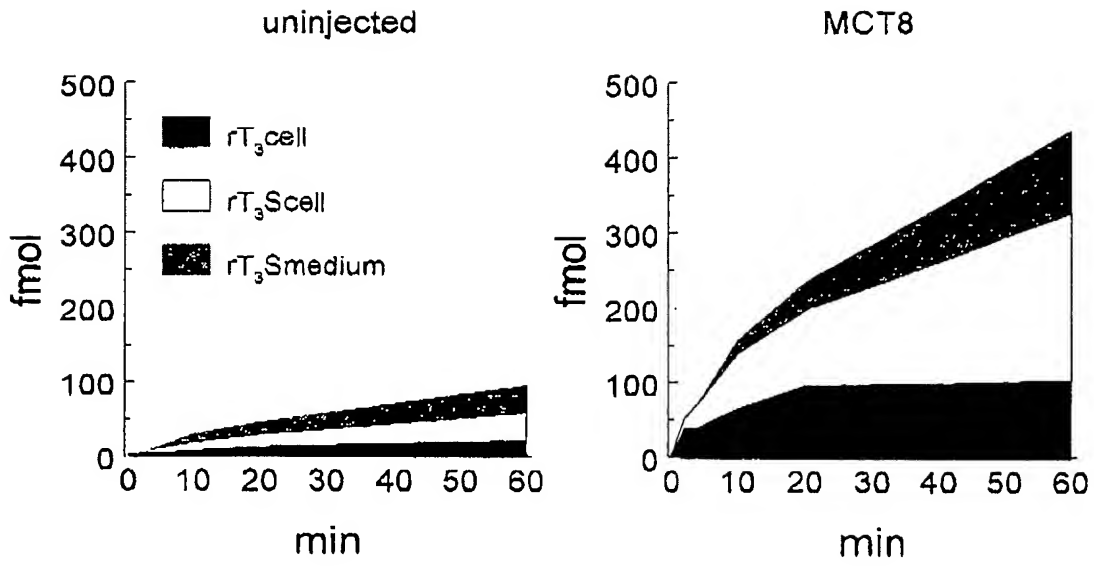
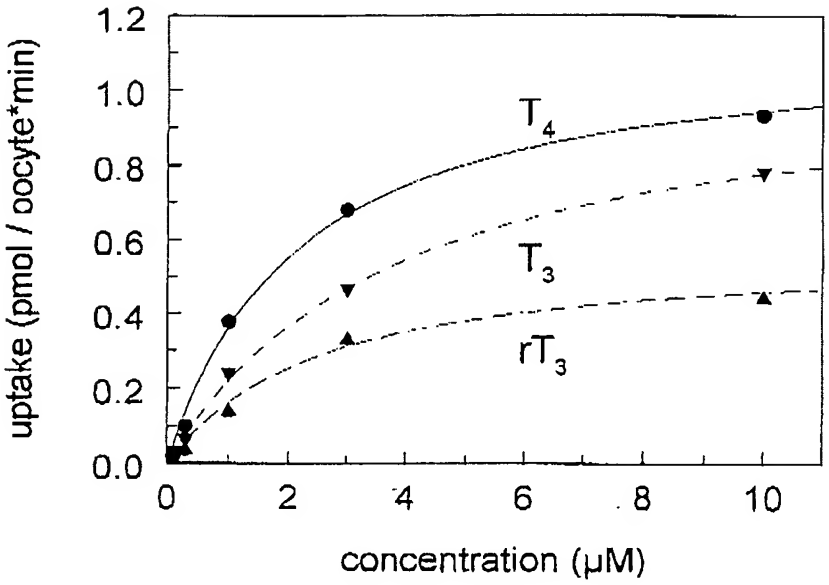

uninjected
MCT8
500
400
300
200
100
0
fmol
rT3cell
rT3Scell
rT3Smedium
0 10 20 30 40 50 60
min

Fig. 14


1.2
1.0
0.8
0.6
0.4
0.2
0.0
uptake (pmol / oocyte*min)
0
2
4
6
8
10
concentration (µM)
T4
T3
rT3

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 03/00384

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/47 A61K38/17 A61K45/00 A61K48/00 G01N33/50
A61P5/14 //A61K38/00,A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 02 22684 A (YUE HENRY;INCYTE GENOMICS INC ; LEE ERNESTINE A (US)) 21 March 2002 (2002-03-21) page 1, line 4 - line 8 page 2, line 22 -page 3, line 6 page 41, line 5 - line 6 page 45, line 29 -page 46, line 27 page 47, line 32 -page 49 *SEQ ID NO: 12; SEQ ID NO: 20* page 53, line 14 ---	1,7,13, 15-17, 19,20, 22,24,25
X	EP 0 982 399 A (STICHTING TOT BEVORDERING VAN) 1 March 2000 (2000-03-01) cited in the application the whole document --- -/--	1,7,13, 19,20, 22,24,25

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 November 2003	11/11/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Bayrak, S

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 03/00384

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	FUJIWARA KOH ET AL: "Identification of thyroid hormone transporters in humans: Different molecules are involved in a tissue-specific manner." ENDOCRINOLOGY, vol. 142, no. 5, May 2001 (2001-05), pages 2005-2012, XP002236843 ISSN: 0013-7227 the whole document	1,7,13, 19,20, 22,24,25
X	WO 99 41373 A (INCYTE PHARMA INC ;CORLEY NEIL C (US); YUE HENRY (US); AU YOUNG JA) 19 August 1999 (1999-08-19) page 23, line 1 - line 10 page 37, line 29 -page 38, line 10 page 52, line 21 -page 53, line 15	1,7,13, 15-17, 19,20, 22,24,25
X	WO 02 06331 A (MEYERS RACHEL ;MILLENNIUM PHARM INC (US)) 24 January 2002 (2002-01-24) page 8, line 11 - line 19 page 14 -page 16 page 75 -page 78	1,7,13, 15-17, 19,20, 22, 24-28,30
X	WO 02 29041 A (CURTIS RORY A J ;MILLENNIUM PHARM INC (US)) 11 April 2002 (2002-04-11) page 5, line 24 - line 25 page 6 -page 10 page 57, line 16 -page 66, line 12	1,7,13, 15-17, 19,20, 22, 24-28,30
X	EP 1 184 465 A (ASTRAZENECA AB) 6 March 2002 (2002-03-06) page 4, line 43 - line 46; example 1 page 5, line 27 - line 28; example 1 page 6, line 30 - line 36 example 1	15,17, 22, 24-28,30
X	HALESTRAP ANDREW P ET AL: "The proton-linked monocarboxylate transporter (MCT) family: Structure, function and regulation" BIOCHEMICAL JOURNAL, PORTLAND PRESS, LONDON, GB, vol. 343, no. 2, 15 October 1999 (1999-10-15), pages 281-299, XP002205906 ISSN: 0264-6021 table 2	22,24,25

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NL 03/00384

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [X] Claims Nos.: 2-4,8-12,14 and 5-7,13,20,24-28,30 (partially)
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 26-29 are directed to a diagnostic method practised on the human/animal body, and claims 14-18, and 1,6,13,19 (as far as in vivo application is concerned) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box I.2

Claims Nos.: 2-4,8-12,14 and 5-7,13,20,24-28,30 (partially)

1. Claims 2-4,8-12,14 and 5-7,13,20,24-28,30 (partially) encompass a genus of compounds defined only by their function wherein the relationship between the structural features of the members of the genus and said function have not been defined ("an isolated molecule capable of specifically binding a monocarboxylate transporter protein or a functional part, derivative and/or analogue thereof, or a ligand of said MCT", or " a compound capable of influencing the binding or transporting of a ligand of, or capable of binding to, a plasma membrane polypeptide capable of transporting a thyroid hormone..."). In the absence of such a relationship either disclosed in the as-filed application or which would have been recognized based upon information readily available to one skilled in the art, the skilled artisan would not know how to make and use compounds that lack structural definition. The fact that one could have assayed a compound of interest using the claimed assays does not overcome this defect since one would have no knowledge beforehand as to whether or not any given compound (other than those that might be particularly disclosed in an application) would fall within the scope of what is claimed. It would require undue experimentation (be an undue burden) to randomly screen undefined compounds for the claimed activity. Therefore, no search has been performed for claims 2,3,4,8-12 and 6,13,14 (partially) (Art. 5 and Art. 6 PCT).

2. Claims 1-16,18-20,22,24-30 relate to compounds defined by reference to a desirable characteristic or property, namely "...a functional part, derivative and/or analogue thereof", "an isolated molecule capable of specifically binding a monocarboxylate transporter protein or a functional part, derivative and/or analogue thereof, or a ligand of said MCT", " a compound capable of influencing the binding or transporting of a ligand of, or capable of binding to, a plasma membrane polypeptide capable of transporting a thyroid hormone, wherein said polypeptide comprises a MCT or a functional part, derivative and/or analogue thereof", "Use of at least a functional part of MCT, or a nucleic acid encoding at least a functional part of a MCT..", "use of at least a functional part of MCT, or a nucleic acid encoding at least a functional part..", "comprises a mutation in a nucleic acid sequence")". The said claims thus cover all compounds having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and disclosure within the meaning of Article 5 PCT for only a very limited number of such compounds. Consequently, the claims lack support and the application lacks disclosure. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

the above reasoning, the claims also lack clarity because the said expressions used in claims 1-4,7,8,13,15,17,19,20,22,24,26,30 are unclear and leave the reader in doubt as to the meaning of the technical feature to which they refer, thereby rendering the definition of the subject-matter of said claims unclear (Article 6 PCT). Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible.

3. Claims 11,14-16,18,26-29 relate to the use of a pharmaceutical preparation for prophylaxis or treatment of "cardiovascular diseases","thyroid hormone related disorder", "disorder of thyroid metabolism", or "non-thyroidal illness" which encompasse a multitude of different diseases. The claims thus cover a rather large number of diseases, whereas the application provides support within the meaning of Article 6 PCT and disclosure within the meaning of Article 5 PCT for only a very limited number of diseases. Consequently, the claims lack support and the application lacks disclosure. Independent of the above reasoning, the claims 15,16,18 also lack clarity because it is not fully possible to determine the diseases for which protection might legitimately be sought (Article 6 PCT). Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible.

Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to monocarboxylate transporter proteins or monocarboxylate transporter protein 8 in relation to the treatment of obesity; and with due respect to the general concept of the invention.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/NL 03/00384

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0222684 A	21-03-2002	AU 9101101 A	26-03-2002
		CA 2422497 A1	21-03-2002
		EP 1326972 A2	16-07-2003
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		US 2003157484 A1	21-08-2003